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54) [Title of the invention]

A cyclodextrin glucanotransferase that is stable in alkali and a method of production thereof.

57) [Summary]

[Aim]
This invention pertains to a cyclodextrin glucanotransferase

and a method of production thereof.

[Construction]

A micro-organism that belongs to the genus Bacillus and that has the capacity of production of cyclodextrin glucanotransferase is cultured, and caused to produce an alkali resistant CGTase in the culture product. This enzyme is used in applications such as detergents.

[What is claimed]

[Claim 1]

A cyclodextrin glucanotransferase that has at least the below mentioned physical and chemical properties.

a) Action: it synthetizes α-, β- and γ-cyclodextrins by hydrolysis of starch,

b) Optimum pH: close to pH 6 (1% soluble starch, 10 mM acetate buffer solution or phosphate buffer solution, 40° C, 30 minutes) c) Optimum temperature: close to 65° C (1% soluble starch, 10 mM phosphate buffer solution (pH 10), 10 minutes)

d) pH stability: pH 5-10 (10 mM acetate buffer solution or phosphate buffer solution, 25° C, 3 hours)

e) Molecular weight: ca. 43,000 (acrylamide electrophoresis method)

f) Inhibitors: it is inhibited by Hg^{2+}, Ag^{+}, Cu^{2+}, Ni^{2+} and Fe^{2+}.

[Claim 2]

A method of production of cyclodextrin glucanotransferase with the characteristic that a micro-organism that belongs to the genus Bacillus and that has the capacity of production of cyclodextrin glucanotransferase that has at least the below mentioned enzymatic and chemical properties, is cultured, and caused to produce the cyclodextrin glucanotransferase in the culture product, and that it is collected.

a) Action: it synthetizes α-, β- and γ-cyclodextrins by hydrolysis of starch,

b) Optimum pH: close to pH 6 (1% soluble starch, 10 mM acetate buffer solution or phosphate buffer solution, 40° C, 30 min-

utes)

c) Action pH: 4-12,

c) Optimum temperature: close to 65° C (1% soluble starch, 10 mM phosphate buffer solution (pH 10), 10 minutes)

d) pH stability: pH 5-10 (10 mM acetate buffer solution or phosphate buffer solution, 25° C, 3 hours)

e) Molecular weight: ca. 43,000 (acrylamide electrophoresis method)

f) Inhibitors: it is inhibited by Hg^{2+}, Ag^{+}, Cu^{2+}, Ni^{2+} and Fe^{2+}.

[Claim 3]

The method of production of cyclodextrin glucanotransferase that has been described in claim 2, wherein the micro-organism is Bacillus sp. YT-1 (FERM P-13877).

[Detailed description of the invention]

[Field of use for the industry]

This invention pertains to a cyclodextrin glucanotransferase (EC 2.4.1.19, below called CGTase) and a method of production thereof. With more details, it pertains to a method of production of CGTase wherein a micro-organism that belongs to the genus Bacillus and that has the capacity of production of CGTase, is cultured, and caused to produce an alkali resistant CGTase in the culture product, and this is collected.

[Existing technology]

Cyclodextrin is a general name for annular oligosaccharides that consist of 6, 7 or 8 glucoses etc., and they are produced from starch by CGTases that are produced by bacteria of the genus Bacillus, such as Bacillus macerans, Bacillus megaterium and Bacillus stearothermophilus, and bacteria such as Klebsiella pneumoniae. Or some basophilic micro-organisms, for instance basophilic Bacillus no. 38-2, produce CGTases with an optimum pH at pH 5.5 and pH 8.5, and produce β-cyclodextrin from starch. Details of the CGTases that are produced by these micro-organisms have been summarized in for instance Fermentation and Industry (Hakko to Kogyo) 36 (3), 176-183 (1978), Brewing (Jozo) 80 (7), 434-440 (1985), 'Basophilic micro-organisms' 101-110

(1982, issued by (Co.) Gakkai Shuppan Center (Academic publication center) etc.

The cyclodextrins that are produced by allowing the various above mentioned CGTases to act on starch, are known to have the actions of encapsulation of various organic compounds, stabilization of instable materials, preservation of fragrances, deodorization of materials with a bad smell, removal of bitterness, promotion of emulsification and improvement of sudsing etc., and they are used in the fields of medicines and food. Methods wherein for instance cyclodextrin is mixed in a detergent, and its sudsing properties are improved (patent disclosure 63-68520, patent disclosure 2-34693, patent disclosure 3-172397), and wherein a lasting effect of perfumes is achieved (patent disclosure 1-185399) are known.

[Problems that should be solved by the invention]

The aim of this invention is to discover micro-organisms that produce CGTases that efficiently produce cyclodextrins with a higher alkali resistance, by looking and screening in a wide range of nature, and to develop novel ways of application such as detergents, for the alkali resistant CGTases that are obtained from the said strains.

[Means to solve the problems]

The present inventors obtained alkali resistant CGTase producing micro-organisms in the wide world of nature, with the result that they observed that the CGTase that is produced by Bacillus sp. YT-1 that was isolated from soil, has an optimum pH in the vicinity of 6, is stable in a wide pH range of 5-12, has the property of producing cyclodextrin in a wide pH range of 4-12, and particularly at pH 5-10, has the property of alkali resistance with an activity of 90% or more of the activity at the optimum pH, and suppresses the production of α-cyclodextrin, and produces β-cyclodextrin and γ-cyclodextrin as the main products at pH 9 or higher.

Because thus the cyclodextrin that is produced by this enzyme under an alkalinity of pH 9 or higher has as main components β-cyclodextrin and γ-cyclodextrin with a large cavity, it was observed that this CGTase demonstrates, in addition to decomposi-

tion and removal of soiling by food, such as starch, also a remarkable effect of masking such as deodorization by the produced cyclodextrin, if it is used in detergents, and that it is extremely useful as a detergent component, for improvement of the cleaning capacity by improvement of the sudsing properties and stimulation of the emulsification of soiling etc. This invention has been performed, based on these findings.

The strain Bacillus sp. YT-1 that is used as an example micro-organism in this invention, is one that has been selected from a large number of micro-organisms that had been isolated from the world of nature for the abvoe mentioned aim. The microbiological properties of Bacillus sp. YT-1 are as mentioned below. Moreover, this strain has been deposited as FERM P-13877 in the laboratory for industrial technology of bio-engineering of the institute for industrial technology.

(1) Morphology: rod-shaped bacteria (width 0.8-1.0 μm, length 3.0-4.0 μm)
(2) Spores: cause mycelium to swell with an egg-shape. Diameter 0.9-1.2 μm, formed at the end or in the center of the mycelium.
(3) Gram dyeing: negative
(4) Motility: +
(5) Oxidase: +
(6) Katalase reaction: +
(7) Indole: -
(8) VP: -
(9) pH in VP medium: 7.8
(10) Lithmus milk reaction: -
(11) Methylene blue reduction: -
(12) Tyrosine decomposition: -
(13) Growth in table salt (5, 7 and 10%): -
(14) Decomposition of urea: -
(15) Utilization of citrate (Christensen): -
(16) OF reaction: -
(17) State with respect to oxygen: aerobic
(18) Decomposition of glucose: -
(19) Decomposition of esculin: +
(20) Sulfate reduction: ±

(21) MacConky(?) growth: -
(22) Decomposition of starch: +
(23) Gelatin: +
(24) Tween 80: -
(25) DNase: +
(26) Phenylalanine: -
(27) Egg yolk: -
(28) Casein: + (18 days)
(29) Temperature of growth (0 C): 10-41 (optimum 32-36)
(30) pH of growth: 7.2-11.5 (optimum 8.0-9.0)
(31) Growth in table salt: 0-1
(32) Nutrient requirement:

biotin	±
nyacin	-
thymine	-
folic acid	-
tryptophan	-

(33) Production of acids from sugars (phenolred half-liquid medium, 21 days)

mannitol	+
saccharose	-
xylose	-
sorbitol	-
salicin	+
arabinose	-
glycerin	-
dulcitol	-
glucose	-
maltose	-
mannose	-
lactose	-

The above mentioned microbiological properties were identified, referring to (1) Bergey's Manual of Determinative Bacteriology, vol. 2 (1986), Williams & Wilkins, USA, (2) N.R. Smith, R.E. Gordon, F.E. Clark (1952) Aerobic Sporeforming bacteria, Agr. Monograph, and (3) R.E. Gordon, W.C. Haynes, C.H. Pang (1973) The Genus Bacillus, Agr. Handbook no. 427, United States department of Agriculture, Washington DC. This micro-organism does not grow in 2% table salt, and also the other properties

are extremely similar to Bacillus brevis, but since it decomposes starch and does not decompose tyrosine, it could not be identified as this bacterium. Since, by the utilization of sugars etc., it does of course not correspond to Bacillus alcalophilus, Bacillus firmus, Bacillus lentus and Bacillus circulans etc., this bacterium was christened Bacillus sp. YT-1.

The outlines of the enzymatic properties of the CGTase that is produced by this micro-organism are shown below.

(a) Action and substrate specificity: It synthetizes α-, β- and γ-cyclodextrin by decomposition of starch. The rate of production of these cyclodextrins at pH 7-8 is that they usually are produced with a rate of 20 to 30 : 60 to 70 : 10 to 15, but at pH 9 or higher, the production of α-cyclodextrin is suppressed, and β-cyclodextrin and γ-cyclodextrin are produced as the main components, and the production rate usually is 0 to 3 : 80 to 85 : 10 to 20.

(b) Action pH and optimum pH: the action pH is 4-12, and the optimum pH, when determined after 30 minutes at 40° C in a 1% soluble starch solution of 10 mM acetate buffer or phosphate buffer, is 6-7, and at pH 5-10, it shows an activity that is 90% of the maximum activity or more (see figure 1).

(c) Action temperature and optimum temperature: the maximum of the action temperature is 80° C, and the optimum temperature, when determined after 10 minutes reaction in a 1% soluble starch solution of 10 mM phosphate buffer (pH 10), is 65° C (see figure 2).

(d) pH stability: When treated 3 hours at 25° C in a 10 mM acetate buffer solution or phosphate buffer solution, it is stable at pH 5-10 (see figure 3).

(e) Heat stability: when heated during 10 minutes in a 10 mM phosphate buffer solution (pH 10), it is stable up to 50° C, but at 60° C, it is 80% inactivated. In the presence of 5 mM calcium ions, 90% or more is retained in case of 10 minutes heating at 60° C (see figure 4).

(f) Molecular weight: the molecular weight that is obtained with the acrylamide electrophoresis method is 43,000.

(g) Stabilization: it is heat stabilized in the presence of Ca^{2+}.

(h) Inhibitors: the activity is inhibited by the presence of

Hg^{2+}, Ag^{+}, Cu^{2+}, Ni^{2+} and Fe^{2+} etc.

(i) Method of purification: by DEAE-cephalose column chromatography and sephadex gel chromatography of the 60% ammoniumsulfate precipitate from the culture supernatant, it can be purified to homogeneity in electrophoresis.

(j) Method of determination of the activity: a proper quantity of enzyme solution was added to 0.2 ml of a 0.1 M phosphate buffer solution (pH 11.0) that contained 2% soluble starch, the total quantity was supplemented to 0.4 ml with water, and this was reacted during 10 minutes at 50° C. After the reaction, 2 ml iodine-potassiumiodide-HCl solution [(addition of water to (0.05 g I_2, 0.05 g KI, 10 ml 0.1 M HCl) to a total quantity of 100 ml], and this was left standing at room temperatrue during 15 minutes, and thereafter colorimetry was executed at 660 nm. The quantity of enzyme that causes a 1% reduction of the colour in 1 minute under these circumstances is 1 unit.

In the past, many micro-organisms that produce cyclodextrins have been discovered. The kind of the produced cyclodextrins depends on the kind of micro-organism, and the enzyme of Bacillus macerans, for instance, produces relatively much α-cyclodextrin (α:β:γ=13.3:2.3:1). In the same way, some bacteria, for instance Bacillus megaterium, produce relatively much β-cyclodextrin (α:β:γ=1.0:7.0:1.0). Moreover, the CGTase that is produced by basophilic bacteria, for instance Bacillus no. 38-2, has its optimum pH as 5.5 and 8.5, and produces β-cyclodextrin [Hakko to Kogyo 37(2), 150-161 (1979)].

The CGTase that is produced by Bacillus sp. YT-1, as the bacterium that is shown as an example in this invention, is stable in a wide pH range of pH 5-10, and has an optimum pH at 6-7, and when it is reacted at pH 6-8, it usually produces 20-30% α-cyclodextrin, 60-70% β-cyclodextrin, and 10-15% γ-cyclodextrin. When it is reacted at pH 9 or higher, on the other hand, the production of α-cyclodextrin is suppressed, and β-cyclodextrin and γ-cyclodextrin with large cavities are produced as the main components. The ratio thereof is 0-3% α-cyclodextrin, 80-85% β-cyclodextrin, and 10-20% γ-cyclodextrin (see table 1). The maximum yield thereof, however, is almost the same, 50-60%.

In the production of the CGTase of this invention by the culture of this micro-organism, organic ntirogen sources such as

soy bean scrap, corn steep liquor, meat extract, pepton, milk casein and yeast extract, are used as nitrogen sources. Among them, soy bean protein, soy bean scrap (defatted soy beans) and corn steep liquor etc. are good nitrogen sources. Besides, if necessary, inorganic nitrogen sources such as ammoniumsulfate, ammoniumchloride, urea and ammoniumnitrate are used. As carbon sources usually starch or starch derivatives such as liquefied starch, soluble starch and dextrin are used.

In addition to the above mentioned nitrogen sources and carbon sources, phosphates, magnesium salts, sodium salts and potassium salts are used as supplementary raw materials. Particularly addition of phosphates, magnesium ions and manganese ions is effective, and as the phosphates, for instance K_2HPO_4 in the order of 0.05-0.3%, as the magnesium salt, for instance $MgSO_4.7\ H_2O$ in the order of 0.01-0.3%, and as the calcium salt, for instance $CaCl_2$ in the order of 0.01-0.1% are added. Since CGTase is produced outside the mycelium, the enzyme is recovered by removing the mycelium after the culture by filtration or centrifugal separation.

Experiments on the resistance of CGTase in detergents with a high alkalinity were carried out with commercial detergents, for instance Mamaroyal, product of Lion Co. (trade name, contains 30% surfactant), at 40° or 50° C in a solution with pH 10 or pH 11, that contained, as the surfactant, for instance 450 ppm. Below, further details of this invention are described in the concrete by examples of execution, but the range of this invention is not limited to these examples of execution.

[Examples of execution]

Example of execution 1.

200 ml of a medium that consisted of 2% (as solids) corn steep liquor, 0.2% K_2HPO_4, 0.2% $MgSO_4.7H_2O$, and $1x10^{-3}$ M $CaCl_2$, were brought in an erlenmeyer flask of 1 liter, and after 15 minutes sterilization at 121% C, Bacillus sp. YT-1 (FERM P-13877) was inoculated, and this was cultured during 3 days at 30° C in a shaken culture with 225 rpm. The CGTase activity of the supernatant that was obtained by centrifugal separation after the culture, was 184 units/ml medium.

To this culture supernatant, ammoniumsulfate was added to 60% saturation, and after collection of the precipitate that was produced, and dialysis, it was supplied to a DEAE-cellosolve CL-68 column that had been buffered with 25 mM tris buffer solution (pH 7.0), and eluted with KCl with a concentration that changed linearly from 0 to 1 M. The CGTase fraction was collected, concentrated, and after dialysis, subsequently gel filtration with a sephadex G-150 column was carried out. The obtained purified enzyme had 2960 units/A_{280}, and from the viewpoint of electrophoresis, it was homogeneous.

Example of execution 2.

To 200 ml supernatant that has been obtained by a culture in the same way as in example of execution 2 (1 ? translator), ammoniumsulfate was added to 60% saturation, and a precipitate was obtained, and the enzyme solution that had been dialyzed with water, was used for an experiment on production of cyclodextrin from starch.

100 mg potato starch, 1.3 ml 0.4 M glycine-sodiumhydroxide buffer solution (pH 7-10), 0.5 ml 0.2 M $CaCl_2$, and 1.25 units CGTase were supplemented to a total quantity of 10 ml with water, and the reaction was carried out at 50° C. In the course of time, fixed quantities were sampled, and by high performance liquid chromatrography, the quantity of the respective cyclodextrins was determined. The obtained results are shown in table 1.

Table 1.

reaction pH	reaction time (hr)	quantities of respective cyclodextrins in the reaction α-CD	β-CD	γ-CD	total CD
7	4	0.0	41.0	7.1	48.5
	8	3.1	46.2	7.9	57.2
	16	6.0	45.2	6.2	58.4
	24	9.6	42.6	5.3	58.0
8	4	0.0	35.9	5.5	41.4
	8	2.8	43.0	6.1	51.9
	16	2.8	47.1	6.1	56.0
	24	3.9	46.6	7.9	58.4
9	4	0.0	40.9	5.9	46.8
	8	0.0	42.5	5.3	47.8
	16	0.0	42.1	5.9	48.0
	24	1.0	43.5	6.0	50.5
10	4	0.0	32.6	3.2	35.3
	8	0.0	40.2	4.5	44.7
	16	0.0	47.2	4.7	51.9
	24	1.3	47.7	5.2	54.2

CD in this table is cyclodextrin. As is clear from table 1, this enzyme effectively produces cyclodextrins in the wide pH range of 7-10 that was tested, and particularly by the reaction at pH 9 or higher, the production of α-CD was suppressed, and β-cyclodextrin and γ-cyclodextrin were produced as the main ones.

Example of execution 3.

In this example of execution, experiments on the stability of the CGTase of this invention in detergent solutions were carried out.

1.0 ml 0.4 M glycine-sodiumhydroxide buffer solution, 0.3 ml CGTase that had been prepared in examples of execution 1 and 2 (381 units) and 3 ml liquid detergent with twice that standard concentration of use [product of Lion Co., trade name Mamaroyal, surfactant (sodium α-olefinsulfonate, fatty acid alkanolamide, polyoxyethylenealkylether etc.) content 30%] were added, supplemented to a total volume of 6 ml with water (surfactant concentration 450 ppm), adjusted at pH 5.0, 7.0 and 10, and left standing at 35° C. In the course of time, fixed quantities were sampled, and the residual activity was determined. The obtained results are shown in figure 5.

In this figure, -o- shows the case of pH 10, -□- shows the case of pH 5, and -●- shows the case of pH 7.

As is clear from the figure, this enzyme is stable against surfactants, and keeps 50% or more of its activity even after 140 hours of contact. Moreover, this enzyme has a higher stability at pH 10 than at pH 5 and pH 7.

Example of execution 4.

In this example of execution, soiling of starch material that has adhered to food ultensils, was assumed, and experiments on the removal of starch soiling by CGTase in a detergent were carried out.

Rice starch (ca. 30 mg) that had been made to a paste, was pasted on a glass plate, and after drying, it was brought in the liquid detergent that had been used in example of execution 4 (3 ? translator), with various concentrations of CGTase, and they were immersed at pH 10 and pH 11, and at 40° C and 50° C. After

15 minutes the supernatants were collected, and after adjustment of pH to 5.0, respectively 0.05 ml of a 100 times diluted commercial Bacillus α-amylase (Thermamyl 6L, produced and sold by Novo Co.) and Aspergillus niger glucoamylase were added, and reacted during 15 minutes at 60° C. The thus produced reducing sugars were quantitatively determined with the Somogyi-Nelson method. The obtained results are shown in figure 6 and figure 7.

Figure 6 is the case of treatment at pH 10, and figure 7 is the case of treatment at pH 11. The quantity of enzyme of the horizontal axis is the quantity of CGTase of this invention that is peresent (x 25 units), and the vertical axis is the relative value (%) of the quantity of starch that had been decomposed and solubilized. In these figures, -●- is the case of 50° C, and -o- is the case of 40° C. In both cases, starch was decomposed and solubilized (converted into dextrin) by the CGTase in the detergent.

[Results of the invention]

This invention pertains to a CGTase that is produced by a micro-organism of the genus Bacillus, and that produces β-cyclodextrin and γ-cyclodextrin at pH 9 or higher. This enzyme is stable in alkali and can be effectively used as a detergent component.

[Brief description of the figures]

[Figure 1]

shows the optimum pH of the CGTase of this invention. In this figure, -●- is the case wherein an acetate buffer solution is used, and -o- is the case wherein a phosphate buffer solution (KH_2PO_4-Na_2HPO_4 or NaOH-Na_2HPO_4) is used.

[Figure 2]

shows the optimum temperature of the CGTase of this invention.

[Figure 3]

shows the pH stability of the CGTase of this invention. In this figure -●- is the case wherein an acetate buffer solution

is used, and -o- is the case wherein a phosphate buffer solution is used.

[Figure 4]

shows the temperature stability of the CGTase of this invention. -o- shows the case wherein no calcium ions are present, and -●- shows the case wherein 5 mM $CaCl_2$ is present.

[Figure 5]

shows the pH stability of the CGTase of this invention in a detergent. In this figure, -o- shows the case of pH 10, -□- shows the case of pH 5, and -●- shows the vcase of pH 7.

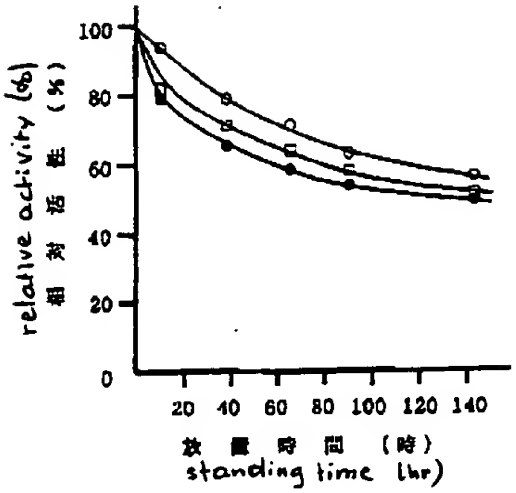
[Figure 6]

shows the action of decomposition and solubilization in the case that starch soiling is treated at pH 10 with the CGTase of this invention that has been added to a detergent. In this figure -●- shows the case of 50° C, and -o- shows the case of 40° C.

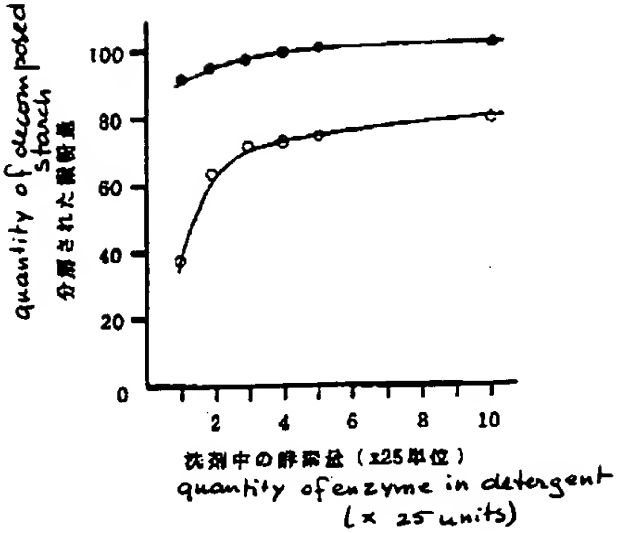
[Figure 7]

shows the action of decomposition and solubilization in the case that starch soiling is treated at pH 11 with the CGTase of this invention that has been added to a detergent. In this figure -●- shows the case of 50° C, and -o- shows the case of 40° C.

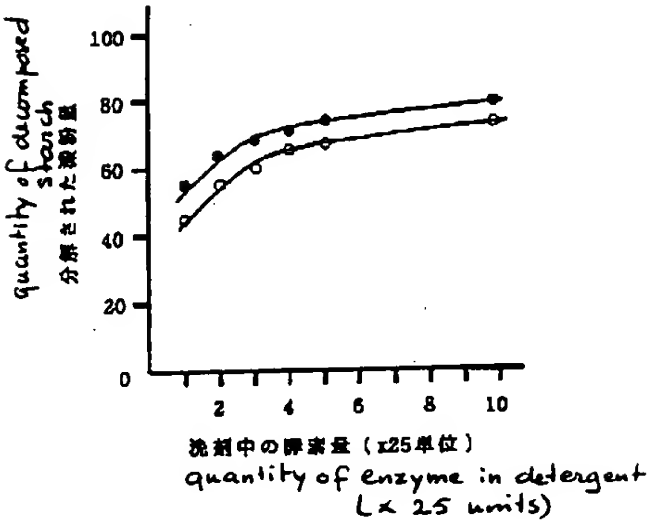
【図5】


relative activity (%)
相対活性（%）
100
80
60
40
20
0
20 40 60 80 100 120 140
放置時間（時）
standing time (hr)

【図6】


quantity of decomposed starch
分解された澱粉量
100
80
60
40
20
0
2 4 6 8 10
洗剤中の酵素量（x25単位）
quantity of enzyme in detergent
(× 25 units)

【図7】


quantity of decomposed starch
分解された澱粉量
100
80
60
40
20
0
2 4 6 8 10
洗剤中の酵素量（x25単位）
quantity of enzyme in detergent
(× 25 units)

9

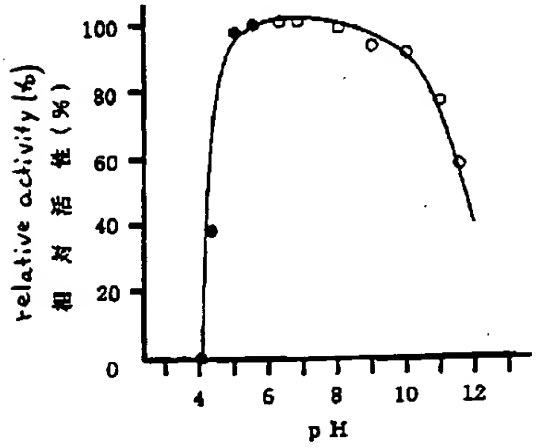
中で－　－は50℃の場合を示し、－○－は40℃の場合を示す。

【図7】洗剤に添加された本発明のCGTaseによる澱粉質

10

汚れのpH11で処理した場合の分解可溶化作用を示す。図中で－　－は50℃の場合を示し、－○－は40℃の場合を示す。

【図1】



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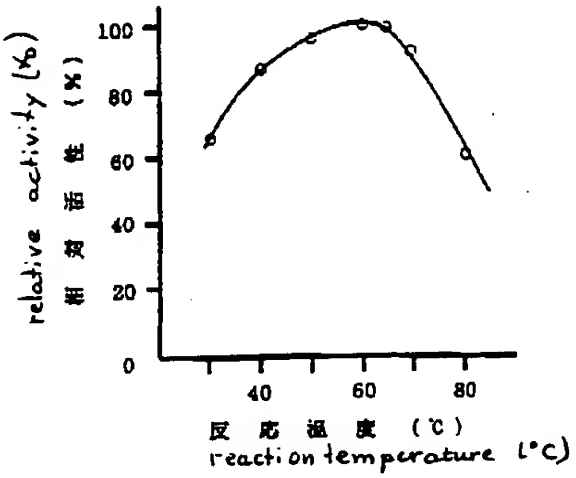
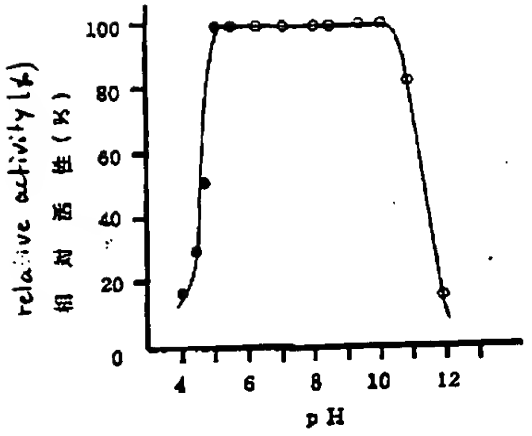


【図3】



【図4】